

Injury to Dorsal Root Ganglia Alters Innervation of Spinal Cord Dorsal Horn Lamina Involved in Nociception

Shin-Ichiro Nakamura, MD, PhD, and Robert R. Myers, PhD

Study Design. A study of the relation between the development of mechanical allodynia and the reorganization of primary afferent terminals in the sensory lamina of the rat spinal cord dorsal horn after partial dorsal root ganglion injury in rats.

Objectives. To investigate the pathologic mechanisms of mechanical allodynia after partial dorsal root ganglion injury.

Summary of Background Data. After experimental peripheral nerve injury causing neuropathic pain, myelinated afferent fibers sprout into lamina II of the dorsal horn. This lamina is associated with nociceptive-specific neurons that generally are not stimulated by myelinated fiber input from mechanical receptors. These morphologic changes are suggested to have significance in the pathogenesis of chronic mechanical allodynia, although it is not known whether this kind of morphologic change occurs after dorsal root ganglion injury.

Methods. After partial dorsal root ganglion crush injury, the mechanical force causing footpad withdrawal was measured with von Frey hairs, and myelinated primary afferents were labeled with cholera toxin B subunit horseradish peroxidase, a selective myelinated fiber tracer that identifies transganglionic synapses.

Results. After partial dorsal root ganglion injury, mechanical allodynia developed in the corresponding footpad within 3 days and persisted throughout the experimental period. At 2 and 4 weeks after the injury, B subunit horseradish peroxidase-positive fibers, presumably myelinated afferents, were observed to be sprouting into lamina II of the dorsal horn on the injured side, but not on the contralateral control side.

Conclusions. Morphologic change in spinal cord dorsal horn lamina II occurs after partial dorsal root ganglion injury. This change may have significance in the pathogenesis of chronic mechanical allodynia after partial dorsal root ganglion injury. [Key words: allodynia, B-HRP, DRG injury, pain, plasticity] **Spine 2000;25:537-542**

Traumatic or degenerative injury to the spine that impinges on the dorsal root ganglion (DRG) can give rise to protracted states of tactile allodynia (*i.e.*, pain to a mechanical stimulus that does not normally provoke pain).¹¹ In posterior decompression surgery, excess ma-

nipulation of the nerve root can cause tactile allodynia, which usually develops within several days and continues for months.

The hypothesis that changes in the neuroanatomic structure of the dorsal horn pain pathways might be involved in the pathogenesis of this state was explored. It is suggested that after rat peripheral nerve transection, myelinated cutaneous A β fibers sprout into lamina II of the dorsal horn, a region consisting mostly of nociceptive specific neurons.^{4,21,27,28} This morphologic change in access to nociceptive neurons by fibers generally considered to mediate nonpainful mechanical information is thought to be one of the pathogenic factors predisposing to neuropathic pain with chronic allodynia. This link has been reported after peripheral nerve transection,²⁷ a condition typically associated with deafferentation pain syndromes. Recently, similar A β central fiber sprouting was seen in some neuropathic pain models such as the Kim and Chung spinal nerve ligation model⁶ and the chronic constriction injury model of sciatic neuropathy.^{14,23}

Cholera toxin B subunit horseradish peroxidase (B-HRP) selectively traces myelinated fibers and can be used to identify their transganglionic terminals.^{5,17,28} To study this phenomenon in the context of spine surgery, the current authors partially crushed rat DRG, then measured the degree of tactile allodynia and the extent of B-HRP-positive fiber sprouting in the ipsilateral and contralateral dorsal horn.

Materials and Methods

All procedures followed the guidelines outlined by the International Association for the Study of Pain and were approved by the Veterans Administration/University of California, San Diego Animal Care Committee.

This study used 20 Sprague-Dawley rats weighing 220–260 g. The rats were housed in pairs with soft bedding, a 12-hour light/dark cycle, and access to food and water *ad libitum*. One group of experimental animals ($n = 8$) was used for 4-week-long behavior measurements of tactile allodynia after partial L4 DRG injury. Other animals received the same DRG injury but were used only for anatomic tracer study at 0 days ($n = 4$), 2 weeks ($n = 4$), and 4 weeks ($n = 4$) after the DRG injury.

The use of separate animals for histologic analysis in neuropathic pain studies reduces the need for behavior testing of very large groups of animals and preserves the integrity of the behavior studies group while minimizing biologic variability. It is agreed generally that animals similarly treated but used only for histologic study at a specific time in the experimental protocol would behavior changes at that time the same as those of animals used for continuous behavior testing.

From the Departments of Anesthesiology and Pathology (Neuropathology), Veterans Affairs Medical Center, San Diego, and the University of California, San Diego, La Jolla, California.

Funded by the Veterans Administration Research Service and National Institutes of Health (NIH) Grant NS 18715-15.

Acknowledgment date: April 2, 1999.

First revision date: May 17, 1999.

Acceptance date: May 28, 1999.

Device status category: 1.

Conflict of interest category: 14.

The L3 and L4 spinal segments were processed for horseradish peroxidase (HRP) histochemistry using the tetramethylbenzidine method. Bilateral L4 DRGs were examined histologically. To help reduce the total number of animals used in this study, four of the animals used for behavior measurements also had B-HRP tracer studies performed on them at the end of the behavior testing period (4 weeks; see later). Spinal cord and bilateral DRGs were examined in three animals, and the profiles of DRG neurons labeled large and small were counted to determine whether significant differences existed in neuronal uptake of the tracer after injury.

Surgical Procedure. A combination of sodium pentobarbital (40 mg/kg) and diazepam (4 mg/kg) was given intraperitoneally and used for anesthesia during surgery. Bilateral L4 DRGs were exposed after L4 laminectomy, and partial L4 DRG injury was created by repeatedly crushing the distal one third of the left DRG with fine forceps. This method has been used previously to characterize the physiologic consequences of DRG crush.¹⁸ The contralateral (right) DRG was used as a control. The wounds were closed, and the rats were allowed to recover in their normal environment.

Behavior Testing. The mechanical sensitivity of the plantar footpad was determined using von Frey filament threshold testing. Brisk withdrawal response to stimulation was determined in experimental animals at 3 consecutive days before and at 1, 3, 5, 7, 10, 14, 21, and 28 days after surgery using the up-down method of Chaplan.²

Tracer Study. For tracing of myelinated fibers, HRP was conjugated to the cholera toxin B-HRP and injected into bilateral L4 DRGs. One group of animals was injected with tracer at the same time they underwent DRG surgery. Other animals were reanesthetized with pentobarbital/diazepam, and their bilateral L4 DRGs were exposed for tracer injection 12 or 26 days after surgery. Using a Hamilton syringe and a 33-gauge needle, 1 mL of 1.5% B-HRP (Sigma, St. Louis, MO) was injected into the proximal one third of the DRG endoneurial space. After 48 hours, terminal anesthesia (intraperitoneal sodium pentobarbital 60 mg/kg and diazepam 6 mg/kg) was administered, and the animals were heparinized (intraperitoneal heparin 1000 U) and then perfused with 250 mL of heparinized saline followed by 500 mL of 1% paraformaldehyde and 1.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at room temperature,¹² and finally 150 mL of 10% sucrose in 0.1 mol/L phosphate buffer (pH 7.4) at 4 C.

Histologic Processing. Because preliminary study showed that B-HRP labeled L3–L4 spinal segments, these spinal segments, determined by dorsal root entry zones, were removed and stored overnight in 30% sucrose in 0.1 mol/L phosphate buffer at 4 C. Every third 40-mm-thick, free-floating, frozen section was processed for HRP histochemistry using the tetramethylbenzidine method,^{12,13} mounted on gelatin-coated slides, dehydrated, cleared in xylene, and coverslipped with Permount.

Serial sections of bilateral L4 DRGs (30 μ m thick) from four animals used for behavior testing were processed by the same method, counterstained with neutral red before dehydration, and viewed by light microscopy for the purpose of counting neurons labeled as having large and small diameters. To avoid

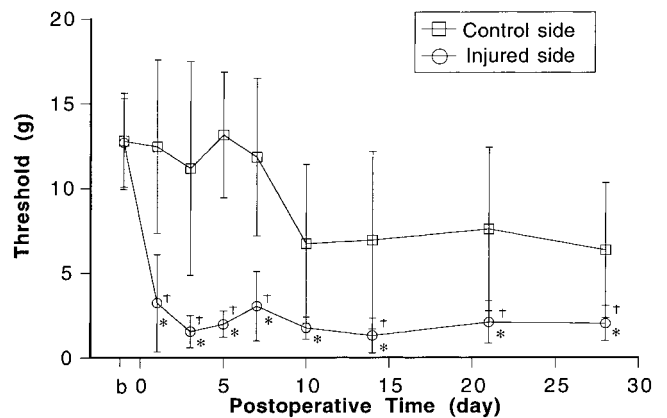


Figure 1. Time course of mechanical sensitivity. Mechanical threshold decreased and reached its minimum at day 3 after partial dorsal root ganglion injury. The mean \pm standard deviation represents the threshold responses of foot withdrawals to von Frey filament stimulation. b represents baseline; $n = 8$; * $P < 0.001$ compared with baseline; † $P < 0.05$ compared with control side.

double counting, only neurons containing a nucleus were counted.

The distinction between large and small neurons was determined by size and the following characteristics of the neuron. A large neuron was defined as more than 40 μ m in diameter with a lightly stained cytoplasm and a centrally located nucleolus. A small neuron was defined as less than 40 μ m in diameter with a darkly stained cytoplasm and a peripherally located nucleolus.¹⁹ Bilateral L4 DRGs of animals used for histologic study were embedded in plastic, and 2-mm-thick sections stained by p-phenylenediamine were examined by light microscopy.

Statistics. Data are expressed as the mean \pm standard deviation. Behavior data were compared within and between groups using repeated measure ANOVA followed by Scheffe *post hoc* analysis. Labeled cell profiles were compared between groups using Welch's *t* test. A *P* less than 0.05 was considered significant.

Results

Behavior Testing

After DRG injury, the animals displayed a significant decrease in mechanical threshold response to von Frey hair stimulation that began 1 day after ipsilateral DRG crush and continued throughout the remaining 4 weeks of observation (Figure 1). The peak of allodynia occurred on day 3 after injury. The control (contralateral) side showed a mild decrease in mechanical threshold response from baseline values, but it was not significantly different from presurgery values. No animals showed paresis.

Tracer Study

In the spinal cord, the control side showed labeling of dorsal horn laminae I, III, IV, and V (Figure 2A, 2C, and 2E). In several sections, a few fibers were found in lamina II. Laminae I and III were labeled at the middle of the L3 spinal segment, and labeling shifted medially to laminae I and III caudally until only laminae IV and V were labeled in the distal part of the L4 segment.

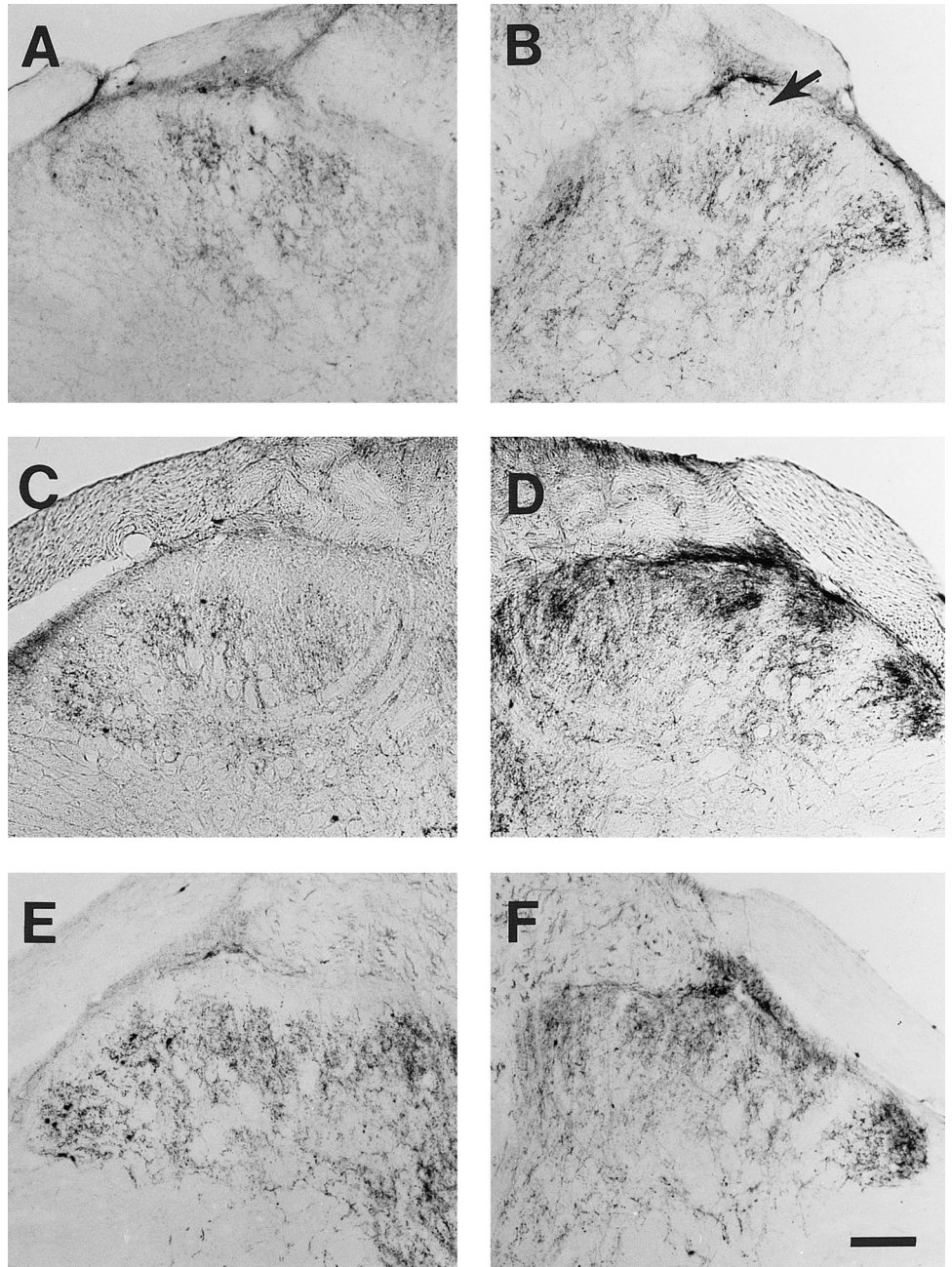


Figure 2. Photomicrographs showing the distribution of the cholera toxin B subunit horse-radish peroxidase (B-HRP) central afferent terminal label in the L3 dorsal horn. **A** and **B**, **C** and **D**, **E** and **F** are corresponding sections of freshly injured tissue 2 and 4 weeks, respectively, after partial dorsal root ganglion injury. In the freshly injured group, both the control (**A**) and injured (**B**) sides clearly are devoid of the B-HRP label in lamina II (arrow). In the 2 weeks after injury group, the control side (**C**) clearly is devoid of the label in lamina II, whereas significant label in lamina II can be seen on the injured side (**D**). In the 4 weeks after injury group, no label in lamina II can be seen on the control side (**E**), but significant label, almost the same degree as in the 2 weeks after injury group, in lamina II can be seen on the injured side (**F**). Bar = 100 μ m.

In dorsal horn lamina ipsilateral to the injured DRG, no difference in labeling existed between control tissue and injured DRG given tracer at the time of DRG injury (Figure 2B). However, every experimental animal studied at 2 or 4 weeks after injury displayed extensive label in lamina II and in laminae I, III, IV, and V of the ipsilateral dorsal horn (Figure 2D and 2F). Although its extent varied in each animal, labeling density of lamina II was as strong as in laminae I and III.

Pathology

The control DRG appeared to be normal, with large-caliber myelinated fibers intermixed with large and small neurons (Figure 3A).

In crushed DRG, the injured area occupied one half to three fourths of the DRG volume. At 2 weeks after in-

jury, edema, degenerated myelinated fibers, and phagocytic cells containing lipid debris were found. By 4 weeks, numerous regenerated small-caliber myelinated fibers also were present in the endoneurium (Figure 3B).

Cell Count of Labeled Neurons

The injured DRG contained 802.5 ± 268.8 labeled large neurons on the average (Figure 4) and 24 ± 6.7 labeled small neurons as counted by light microscopy in 30- μ m-thick sections. The small neurons labeled per total neurons labeled was $3.2\% \pm 1.1\%$. The control DRG contained 970.5 ± 439.5 labeled large neurons on the average and 28.3 ± 13.7 labeled small neurons. The small labeled neurons per large neurons labeled was $2.9\% \pm 0.3\%$. Neither of these changes was significant.

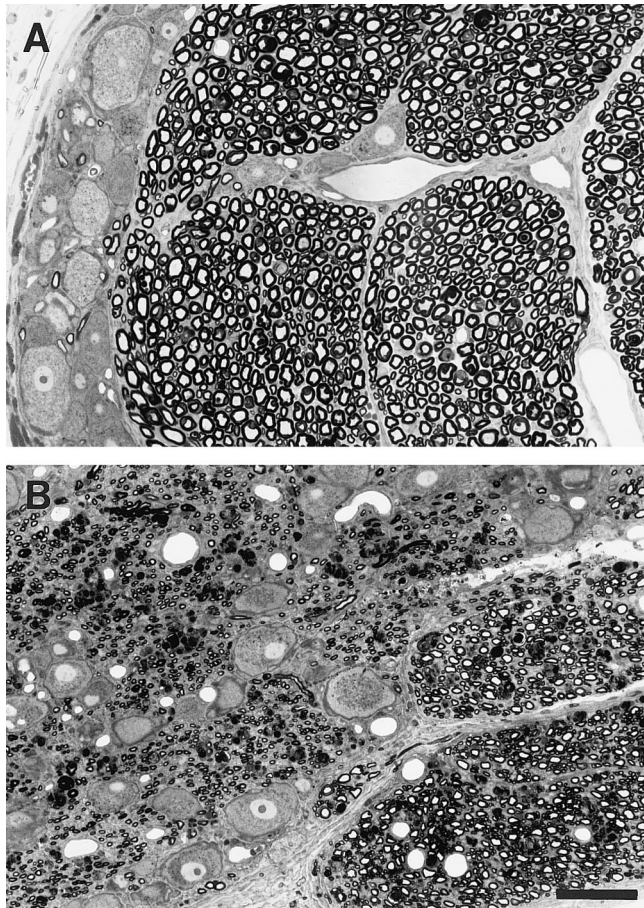


Figure 3. Photomicrograph showing L4 dorsal root ganglia 4 weeks after the injury. Many large-caliber myelinated fibers and neurons can be seen on the control side (A). On the injured side, many of the large-caliber myelinated fibers are degenerated, and many regenerated small-caliber myelinated fibers can be seen. However, there still are intact fibers and neurons (B). p-phenylenediamine staining. Bar = 100 μ m.

Discussion

Neuroanatomic methods have been used repeatedly to explore relations between peripheral function and central structure. Of particular interest to spine surgeons are the central mechanisms through which injury to the dorsal root ganglion gives rise to tactile allodynia (*i.e.*, the pain caused by innocuous stimulation of peripheral mechanoreceptors). Several experiments discussed later have suggested that the central axonal projections from DRG neurons associated with innocuous proprioception gain access to nociceptive neurons in the spinal cord dorsal horn after severe nerve injury, and that this change may be related to the pathogenesis of mechanical allodynia. The question asked in this study was whether DRG crush injury causes these anatomic changes in the central innervation of pain structures. Specifically, the authors measured behavior responses to peripheral mechanical stimulation and asked whether DRG crush injury causes cholera toxin B-HRP-positive fibers to sprout into the ipsilateral dorsal horn lamina II.

The B subunit of cholera toxin conjugated to B-HRP has been used widely as a retrogradely transported marker to study transganglionic axonal terminations. Originally used by Stoessel²⁴ in retrograde axonal transport studies, B-HRP is known to bind to GM1 ganglioside (a constituent of only myelinated axons) and to label myelinated fibers, large DRG neurons, and their primary afferent terminals in rat dorsal horn.^{5,17} More recently, B-HRP has been used extensively by Woolf et al^{27,28} to study the changes in dorsal horn innervation after peripheral nerve axotomy, and by others to study less severe injuries to nerve fibers that give rise to allodynic pain states.^{6,14,23,27}

In the rat, myelinated afferent fibers terminate predominantly in lamina I (A δ) and in laminae III, IV, and V (A δ and A β) of the dorsal horn, whereas unmyelinated primary afferent fibers terminate primarily in lamina II,^{7,8,10,16,20,22,25} a region consisting mostly of nociceptive specific neurons²⁷ integral to pain processing. Using retrograde transganglionic tracing and other techniques, it has been concluded that nerve axotomy induces sprouting of myelinated primary afferent fibers into lamina II of the dorsal horn.^{27,28} Moreover, it is postulated that this structural reorganization contributes to the development of allodynia that begins shortly after the injury and persists for months.

In this study, these results are extended, showing that similar changes in B-HRP labeling of dorsal horn lamina II occurs after experimental DRG injury. Furthermore, these changes are associated with the development of mechanical allodynia displayed in a temporal pattern consistent with the hypothesized link between myelinated fiber sprouting in lamina II and the development of allodynia.

In the current study, B-HRP injected into control DRG showed labeled terminals only in laminae I, III, IV, and V, a finding consistent with other reports.^{6,14,23,27} The data also indicate that there was no direct uptake by unmyelinated small neurons that terminate in lamina II

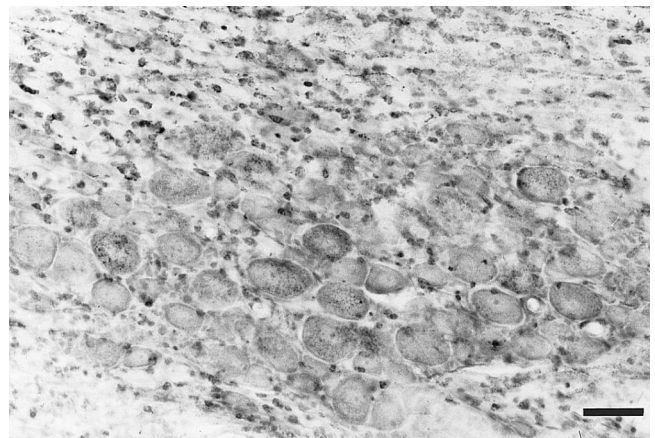


Figure 4. Photomicrographs showing the labeled neurons on the injured side of L4 dorsal root ganglia 4 weeks after the injury. Most of the labeled neurons are large neurons. Bar = 100 μ m.

after injection of the tracer directly into normal DRGs. There was no label in lamina II of animals that had B-HRP injected just after DRG injury. There also was no significant difference between the proportion of labeled small neurons and that of labeled large neurons between injured and control DRG 4 weeks after the injury. However, after DRG crush injury, sprouting of B-HRP-positive fibers was noted in the ipsilateral dorsal horn lamina II 2 and 4 weeks after injury. These data support the Woolf et al^{27,28} hypothesis.

This hypothesis is appealing because myelinated A β fibers signal vibratory sense, light touch, and position sense in normal skin, but not painful stimuli.¹⁵ Behavior and pharmacologic studies of mechanical allodynia using differential nerve blocks with local anesthetics and limb compression have emphasized uniformly that allodynia is mediated by the activation of low-threshold, rapidly conducting, and presumably large primary afferents (A β fibers).^{1,3,9} This hypothesis is based on the presumed functional connections of A β sprouts with neurons in lamina II shown to occur after nerve transection.²⁸

At 2 weeks after axotomy, 15 times more synaptic terminals in lamina II than normal were labeled by B-HRP. These sprouted A β fibers evoked monosynaptic cord dorsum potentials in lamina II neurons similar in configuration to those produced by identified nociceptors in control tissue.⁴ It also was shown that there was facilitation in response to the conditioning–testing pairs paradigm. As further support for the hypothesis, these sprouts into lamina II remained more than 6 months after the axotomy and did not degenerate after peripheral fibers reinnervated the periphery.²⁸ Although behavior and histologic observations in this study were limited to 4 weeks, this time course of sprouting is consistent with the long-lasting character of allodynia associated with DRG injury.

A recent report, however, questions the underlying assumptions of these associations and demonstrates that the cholera toxin B subunit may be taken up by injured unmyelinated axons in the rat and by small unmyelinated axons found normally in the monkey.²⁶ In the normal monkey, cholera toxin B subunit labeling is seen primarily in dorsal horn laminae I and II, whereas in the rat, it is confined largely to laminae III to V. After injury to monkey axons, small DRG neurons take up the tracer. This also may occur in the rat to a greater degree than had been appreciated originally.²⁶

Although it is difficult at this time to reconcile these apparent conflicting neuroanatomic studies, it is clear from the current experiments that rat DRG injury causes mechanical allodynia as well as B-HRP axonal uptake and transport to dorsal horn lamina II. This occurs in a way that differs from the control state, with function-specific segregation of afferent input to specific dorsal horn laminae. Further study on the relation between structure and function should provide information to

resolve these possible discrepancies and improve our understanding of the neuroanatomic basis of pain.

■ Key Points

- Injury to a dorsal root ganglion (DRG) is often associated with chronic sensitivity to mechanical stimulation of the associated dermatome.
- Dorsal root ganglion neurons are the primary sensory neurons of the peripheral nervous system.
- The superficial dorsal horn lamina of the spinal cord contain second-order neurons with synaptic connections from DRG neurons organized primarily by sensory function.
- After DRG injury, there is anatomic evidence that peripheral nerve fibers conveying proprioceptive information sprout into the dorsal horn lamina (II) associated with nociception.
- These anatomic changes may relate to the development of mechanical allodynia after DRG injury.

Acknowledgment

The authors thank Heidi M. Heckman, Joanne Steinauer, and Jenny Dolkas for their expert technical assistance.

References

1. Campbell JN, Raja SN, Meyer RA, Mackinnon SE. Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain* 1988;32:89–94.
2. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Method* 1994;53:55–63.
3. Gracely RH, Lynch SA, Bennett GJ. Painful neuropathy: Altered central processing maintained dynamically by peripheral input. *Pain* 1992;51:175–94.
4. Koerber HR, Mirnic K, Brown PB, Mendell PB. Central sprouting and functional plasticity of regenerated primary afferents. *J Neurosci* 1994;14:3655–71.
5. LaMotte CC, Kapadia SE, Shapiro CM. Central projections of the sciatic, saphenous, median, and ulnar nerves of the rat demonstrated by transganglionic transport of cholera toxin B subunit (B-HRP) and wheat germ agglutinin-HRP (WGA-HRP). *J Comp Neurol* 1991;311:546–62.
6. Lekan HA, Carlton SM, Coggeshall RE. Sprouting of A- β fibers into lamina II of the rat dorsal horn in peripheral neuropathy. *Neurosci Lett* 1996;208:147–50.
7. Light AR, Perl ER. Reexamination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. *J Comp Neurol* 1979;186:117–32.
8. Light AR, Perl ER. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. *J Comp Neurol* 1979;186:133–50.
9. Lindblom U, Verrillo RT. Sensory functions in chronic neuralgia. *J Neurol Neurosurg Psychiatry* 1979;42:422–35.
10. Maxwell DJ, Réthelyi M. Ultrastructure and synaptic connections of cutaneous afferent fibres in the spinal cord. *TINS* 1987;10:117–23.
11. Merskey H, Lindblom U, Mumford JM, Nathan PW, Noordenbos W, Sunderland S. Pain terms: A current list with definitions and notes on usage. *Pain* 1986;3(Suppl):S215–21.
12. Mesulam M-M. Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: A noncarcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. *J Histochem Cytochem* 1978;26:106–17.
13. Mesulam M-M, Hegarty E, Barbas H, et al. Additional factors influencing sensitivity in the tetramethyl benzidine method for horseradish peroxidase neurohistochemistry. *J Histochem Cytochem* 1980;28:1255–9.
14. Nakamura S, Myers RR. Myelinated afferents sprout into lamina II of L3–L5 dorsal horn following chronic constriction nerve injury in rats. *Brain Res* 1999;818:285–90.

15. Pierce PA, Brose WG. Causalgia/reflex sympathetic dystrophy. In: Yaksh TL, Lynch C, Zapol W, Maze M, Biebuyck JF, LJ S, eds. Anesthesia: Biologic Foundations. Philadelphia: Lippincott-Raven, 1997:889-904.
16. Réthelyi M, Light AR, Perl ER. Synaptic complexes formed by functionally defined primary afferent units with fine myelinated fibers. *J Comp Neurol* 1982; 207:381-93.
17. Robertson B, Grant G. A comparison between wheat germ agglutinin- and cholera toxin B-subunit as anterogradely transported markers in central branches of primary sensory neurons in the rat with some observations in the cat. *Neuroscience* 1985;14:895-905.
18. Rydevik BL, Myers RR, Powell HC. Pressure increase in the dorsal root ganglion following mechanical compression: Closed compartment syndrome in nerve roots. *Spine* 1989;14:574-6.
19. Schmalbruch H. The number of neurons in dorsal root ganglia L4-L6 of the rat. *Anat Rec* 1987;219:315-22.
20. Shortland P, Woolf CJ. Chronic peripheral nerve section results in a rearrangement of the central axonal arborizations of axotomized A beta primary afferent neurons in the rat spinal cord. *J Comp Neurol* 1993;330:65-82.
21. Shortland P, Woolf CJ. Morphology and somatotopy of the central arborizations of rapidly adapting glabrous skin afferents in the rat lumbar spinal cord. *J Comp Neurol* 1993;329:491-511.
22. Shortland P, Woolf CJ, Fitzgerald M. Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. *J Comp Neurol* 1989;289:416-33.
23. Shortland P, Kinman E, Molander C. Sprouting of A-fibre primary afferents into lamina II in two rat models of neuropathic pain. *Eur J Pain* 1997;1:215-27.
24. Stoessel K, Schwab M, Thoenen H. Role of gangliosides in the uptake and retrograde transport of cholera and tetanus toxin as compared to nerve growth factor and wheat germ agglutinin. *Brain Res* 1977;132:273-85.
25. Sugiura Y, Lee CL, Perl ER. Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. *Science* 1986;234:358-61.
26. Tong Y-G, Want F, Ju G, Grant G, Hokfelt T, Zhang X. Increased uptake and transport of cholera toxin B-subunit in dorsal root ganglion neurons after peripheral axotomy: Possible implications for sensory sprouting. *J Compar Neurol* 1999;404:143-58.
27. Woolf CJ, Shortland P, Coggeshall RE. Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 1992;355:75-8.
28. Woolf CJ, Shortland P, Reynolds M, Ridings J, Doubell T, Coggeshall R. Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. *J Comp Neurol* 1995;360:121-34.

Address reprint requests to

Robert R. Myers, PhD
Department of Anesthesiology (0629)
University of California, San Diego
9500 Gilman Drive
La Jolla, CA 92093-0629
E-mail: rmyers@ucsd.edu